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## Role of FNR and FNR-regulated, sugar fermentation genes in *Neisseria meningitidis* infection

Erika Bartolini<sup>1</sup>, Elisabetta Frigimelica<sup>1</sup>, Serena Giovinazzi<sup>1</sup>, Giuliano Galli<sup>1</sup>, Yazdani Shaik<sup>2</sup>, Caroline Genco<sup>2</sup>, Jo Anne Welsch<sup>3</sup>, Dan M. Granoff<sup>3</sup>, Guido Grandi<sup>1,\*</sup>, and Renata Grifantini<sup>1</sup>

<sup>1</sup>Chiron Vaccines, Via Fiorentina 1, 53100 Siena, Italy

<sup>2</sup>Department of Medicine, Section of Infectious Diseases, 650 Albany Street and Department of Microbiology, Boston University School of Medicine, Boston, MA 02118, USA

<sup>3</sup>Children's Hospital Oakland Research Institute, 5700 Martin Luther King, Jr. Way, Oakland, CA 94609, USA

### Summary

While it is generally accepted that anaerobic metabolism is required during infection, supporting experimental data have only been described in a limited number of studies. To provide additional evidence on the role of anaerobic metabolism in bacterial pathogens while invading mammalian hosts, we analysed the effect of the inactivation of FNR, the major regulatory protein involved in the adaptation to oxygen restrictive conditions, and of two of the FNR-regulated genes on the survival of *Neisseria meningitidis* serogroup B (*MenB*) *in vivo*. We found that *fnr* deletion resulted in more than 1 log reduction in the meningococcal capacity to proliferate both in infant rats and in mice. To identify which of the FNR-regulated genes were responsible for this attenuated phenotype, we defined the FNR regulon by combining DNA microarray analysis and FNR–DNA binding studies. Under oxygen-restricted conditions, FNR positively controlled the transcription of nine transcriptional units, the most upregulated of which were the two operons NMB0388-*galM* and *mapA-pgmβ* implicated in sugar metabolism and fermentation. When *galM* and *mapA* were knocked out, the mutants were attenuated by 2 and 3 logs respectively. As the operons are controlled by FNR, from these data we conclude that *MenB* survival in the host anatomical sites where oxygen is limiting is supported by sugar fermentation.

### Introduction

During infection bacterial pathogens encounter host anatomical sites with low oxygen levels, a condition often exacerbated by pathological events such as ischaemia, oedema and inflammation, triggered by the infection process. The fact that most pathogens have anaerobic pathways capable of providing energy and eventually using nitrate and fumarate as final electron acceptors strongly suggests that anaerobic metabolism plays an important role in the infection process. However, while the interplay between oxygen availability and expression of virulence factors has been well documented in a number of reports (Leclerc *et al.*, 1998; Landini and Zehnder, 2002; Krishnan *et al.*, 2004), the experimental data supporting the requirement of anaerobic metabolism for *in vivo* infection are limited to relatively few studies. These studies include: the observation that the *arcA* and fumarate reductase genes were upregulated in *Vibrio cholera* isolated from stool samples of infected patients (Bina *et al.*, 2003; Xu *et al.*, 2003); the inability of *Salmonella typhi* and *Mycobacterium bovis* mutants

\*For correspondence. E-mail [guido\\_grandi@chiron.com](mailto:guido_grandi@chiron.com); Tel. (+39) 577 243 506; Fax (+39) 577 278 514.

defective in nitrate reductase activity to invade and proliferate in epithelial cells (Contreras *et al.*, 1997) and to persist in organs of infected mice respectively (Fritz *et al.*, 2002); the attenuation of virulence in the animal models of infection observed in *Bordetella pertussis* and *Actinobacillus pleuropneumoniae* mutants in which the *fnr* homologous genes, *btr* and *hlyX*, were deleted (Wood *et al.*, 1998; Baltes *et al.*, 2005). As FNR (Fumarate and Nitrate reductase Regulator protein) (Guest *et al.*, 1996) is the main player in the metabolic switch from aerobic to anaerobic growth, these latter studies suggest that genes involved in anaerobic metabolism may be crucial for bacterial infection.

To further investigate on the link between anaerobic metabolism and bacterial infection and pathogenesis, we used the human pathogen *Neisseria meningitidis* as a model system. This microorganism is capable of adapting to different anatomical compartments of the host, including the nasopharyngeal mucosa, the bloodstream and the subarachnoid compartment (Nassif *et al.*, 1999), sites where oxygen concentrations are expected to fluctuate. Therefore, it is expected that the organism is endowed with a fine-tuned regulation of expression of the aerobic and anaerobic metabolism genes and that mutations that impaired the capacity of the pathogen to cope with the oxygen-limiting conditions interfere with the infection process.

In the present article, we first show that, as it is the case for *B. pertussis* and *A. pleuropneumoniae*, mutations in the aerobic/anaerobic metabolism regulator *fnr* result in an attenuated phenotype. Furthermore, by whole genome transcription analysis we demonstrate that the *mapA* and *galM* genes, involved in sugar fermentation, are regulated by FNR and that their inactivation impairs the ability of *N. meningitidis* serogroup B (*MenB*) to proliferate in the mouse.

## Results

### FNR knockouts mutants are attenuated *in vivo*

To study the role of anaerobic metabolism in *N. meningitidis* pathogenesis, we first asked whether the deletion of the *fnr* gene affects *N. meningitidis* survival *in vivo*. The *fnr* gene was replaced with an *erm* cassette in two different *N. meningitidis* strains (MC58 and BZ232) to be used in an adult mouse model and an infant rat model (Wilks *et al.*, 1998; Granoff *et al.*, 2001) of *N. meningitidis* infection respectively. Under aerobic conditions, the growth rate of both MC-*fnrKO* and BZ-*fnrKO* mutants was comparable to the wild-type strains. This is consistent with the fact that FNR is inactive in the presence of oxygen (Khoroshilova *et al.*, 1997). In contrast, when the mutants were cultured under oxygen restriction, the growth rate, as judged by viable cell counting, was reduced by twofold (data not shown). To analyse the effect of *fnr* deletion *in vivo*, 6-week-old mice were challenged with  $10^7$  colony-forming units (cfu) of either *N. meningitidis* MC-*fnrKO* or the isogenic wild-type strain. Eighteen hours after challenge, the animals were bled and bacteria counted by colony plating. While the wild type reached a bacteraemia of approximately  $10^5$  cfu ml<sup>-1</sup>, the number of viable bacteria of the MC-*fnrKO* deletion mutant was reduced by more than one order of magnitude (Table 1). A similar trend in the reduction of viable bacteria was obtained in the infant rat model using the BZ232 wild-type and BZ-*fnrKO* strains (Table 1).

### Identification of the FNR regulon by DNA microarray analysis

To determine which of the FNR-regulated genes might be responsible for the attenuated phenotype of the strains carrying the *fnr* gene deletion, we utilized microarray analysis to define the FNR regulon of *N. meningitidis*. Global transcription profiling was performed on RNA samples extracted at different time points from both *N. meningitidis* MC58 and MC-*fnrKO* strains cultured in GC medium under oxygen-limiting conditions. Comparison of the gene expression profiles revealed that a group of 11 genes was upregulated by more than twofold

in the wild-type strain and, in general, maintained an upregulated trend throughout the entire time-course of the analysis (Fig. 1A). The 11 genes were organized in nine putative transcriptional units. The two putative operons NMB0388-*galM* and *mapA-pgmβ*, whose operon organization was confirmed by reverse transcription polymerase chain reaction (RT-PCR) analysis (see Fig. S1 in *Supplementary material*), are involved in maltose catabolism (Qian *et al.*, 1994) and intervene in the first steps of the degradation of simple sugars to form pyruvate. The *aniA* and the *nosR* genes, respectively, code for a nitrite reductase (AniA), which reduces nitrite to nitric oxide, and for a putative nitrous oxide reductase regulator that in *Pseudomonas* regulates the expression of nitrous oxide reductase (Arai *et al.*, 2003). The NMB1805 and NMB1677 genes encode cytochrome *c4* and cytochrome *c5*, respectively, belonging to the *c*-type cytochrome family. Finally, the last three genes of the group, NMB0363, NMB1806 and NMB1870, code for hypothetical proteins.

After 3 h of growth under oxygen limitation, the difference in gene expression between the wild-type and mutant strains became much more pronounced. A total of 175 genes were differentially transcribed by more than twofold (Table S1 in *Supplementary material*), of which 126 were upregulated and 49 were downregulated. Although not further investigated, this phenomenon could be in part ascribable to the pleiotropic effect of prolonged oxygen starvation of the mutant strain, in which the major regulator of anaerobic metabolism is missing.

### Confirmation of the FNR regulon by computational and biochemical analyses

To confirm the involvement of FNR in the regulation of the 11 genes identified by microarray analysis, we first scanned the promoter-proximal regions (400 nucleotides upstream from the respective start codons) of the nine transcriptional units belonging to the putative FNR cluster. In all nine transcriptional units, conserved motifs were found whose alignment generated the TTTACTTGATTTAAATTA consensus sequence (Fig. 1B and Fig. S2 in *Supplementary material*). This sequence includes a highly conserved TTGATT TAAATTAA sequence that differs by one nucleotide from both the *Escherichia coli* FNR box (TTGAT-N4-ATCAA) (Guest *et al.*, 1996) and the FNR-binding sequence of the *aniA* gene of *Neisseria gonorrhoeae*, the only FNR box so far characterized in *Neisseria* (Lissenden *et al.*, 2000). When the TTGATTTAAATTAA sequence was searched for in the upstream promoter regions of the 175 genes differentially expressed after 3 h of oxygen starvation (up to four-nucleotide differences accepted), it was found in approximately 30% of the genes (Fig. 1C). A comparable frequency was estimated by scanning all *N. meningitidis* annotated transcriptional units (Tettelin *et al.*, 2000; data not shown). This indicates that the regulatory events directly controlled by FNR are likely to occur early after oxygen starvation and involve the nine clustered transcriptional units, all carrying an FNR consensus sequence. More complex regulatory pathways, not directly dependent on FNR, intervene after prolonged oxygen restriction.

To further support our hypothesis that the nine transcriptional units are under the direct control of FNR, we analysed the capacity of purified FNR to bind to the upstream promoter-proximal regions. Both the oxygen-resistant *E. coli* FNR D154A mutant protein (Ec FNR) (Bates *et al.*, 1995) and the wild-type *N. meningitidis* FNR (Nm FNR) were utilized in electrophoretic mobility shift assay (EMSA) analysis. The Ec FNR was used to take advantage of its oxygen-resistant property so that these experiments could be carried out under normal laboratory conditions. Likewise, this allowed us to investigate the ability of the *E. coli* FNR to recognize *N. meningitidis* FNR binding sites. The Ec FNR was able to bind to five of the nine upstream regions, showing the highest affinity to the promoter-proximal regions of the *aniA* gene and the NMB0388-*galM* gene locus (Table S2 in *Supplementary material*). In contrast, the recombinant Nm FNR was capable of binding all nine upstream regions, although with an apparent lower affinity (Table S2). However, this was likely due to the partial inactivation of

the highly oxygen sensitive Nm FNR, even if particular care was taken to run the experiments under anaerobic conditions. As examples, Ec FNR and Nm FNR binding to the promoter regions of *aniA*, *NMB0388-galM* and *mapA-pgmβ* transcriptional units is shown in Fig. 2. Nm FNR specifically interacted with all three upstream promoter regions when a protein concentration of 800 nM was used. In the case of the *aniA* fragment, Nm FNR interaction also occurred at 400 nM but the band shift was less pronounced, suggesting that the *aniA* region carries two FNR binding sites recognizing the regulatory protein with different affinities. Indeed, inspection of the DNA sequence of the fragment revealed the presence of two putative consensus FNR boxes located 253 nucleotides apart (data not shown). The fact that the Nm FNR–DNA complexes appeared like smears on gel is likely to be due to a partial inactivation of the regulatory protein during the electrophoresis run. In the case of Ec FNR, while no interaction was observed with the *mapA-pgmβ* promoter region, a strong binding to both *aniA* and *NMB0388-galM* fragments occurred at a protein concentration as low as 50 nM.

The specificity of FNR binding to the upstream promoter regions was confirmed by EMSA competition experiments using both the Ec FNR and the Nm FNR and all the DNA fragments that showed affinity to the regulatory proteins. In these experiments, FNR binding to the labelled DNA fragments was followed in the presence of increasing amounts of corresponding unlabelled DNA. Under these conditions, 10-fold excess of unlabelled probes almost completely abolished the FNR binding to the labelled probes (Fig. 2 and data not shown).

Taken together, DNA microarray, bioinformatics and DNA binding studies consistently led to the conclusion that the nine transcriptional units are under the direct control of FNR.

### ***galM* and *mapA* mutants are attenuated *in vivo***

As *galM* and *mapA-pgmβ* are important genes for sugar metabolism under anaerobic conditions, we asked what role *galM* and *mapA* genes might have on meningococcal virulence.

To this aim, the genes were replaced with the *erm* cassette integrated in the chromosome in the opposite orientation with respect to original gene orientation (Fig. 3). RT-PCR was used to confirm each gene deletion. As expected, the replacement of *galM* and *pgmβ* genes resulted in the abrogation of the corresponding transcription products (Fig. 3, panels 2 and 3). We also analysed whether each gene inactivation had affected the transcription levels of the adjacent genes. As shown in Fig. 3, panel 1, *galM* deletion did not influence the transcription of the NMB0388 upstream gene, while marginally affected the transcription level of the *mapA* downstream gene (Fig. 3, panel 3). In the case of *mapA* gene replacement, the transcription levels of the downstream *pgmβ* gene and, marginally, of the upstream *galM* gene were both reduced. These secondary effects were likely due to the transcription activity of the *erm* promoter, which transcribes in the opposite direction with respect to the inactivated transcriptional units (Fig. 3).

In conclusion, RT-PCR analyses confirmed each gene replacement. In the case of *mapA* inactivation, *erm* insertion resulted in a slight reduction in the mRNA levels of the neighbouring genes while *galM* gene substitution only marginally affected the transcription level of the *mapA* downstream gene. These effects should be taken into account for the correct interpretation of the subsequent phenotypic analyses.

Inactivation of both *mapA* and *galM* genes did not affect the growth kinetics of the two mutants in rich medium or in glucose-containing synthetic medium (Fig. S3A in *Supplementary material*). In contrast, both mutants showed an impaired capacity to grow in a chemically defined medium containing maltose as the main carbon source. This is consistent with the fact that the two genes are involved in maltose utilization (Fig. S3B in *Supplementary material*).

We next examined the ability of the individual *N. meningitidis* mutants to proliferate and survive following intraperitoneal injection in the mouse model of infection. As shown in Table 2, the levels of *galMKO* and *mapAKO* mutants in blood were reduced by 2 logs and 3 logs, respectively, as compared with the wild-type strain, suggesting that maltose utilization represents an important energy source for the *in vivo* growth of *N. meningitidis*.

### Complementation of *fnr*, *galM* and *mapA* deletion mutants

To confirm that the attenuated phenotype of the *mapAKO* and *galMKO* mutants was specifically ascribable to the deleted genes, we complemented each mutant by inserting a functional copy of *fnr*, *mapA* and *galM* into the chromosome of MC-*fnrKO*, *mapAKO* and *galMKO*, respectively, between the convergent genes NMB1428 and NMB1429. From previous experience, we knew that gene insertions in this locus did not result in appreciable phenotypic effects (Grifantini *et al.*, 2004). RT-PCR analysis of RNA samples obtained from the resulting three genetically complemented strains *fnrKO\_C*, *mapAKO\_C* and *galMKO\_C* revealed that the transcription levels of *fnr*, *mapA* and *galM* in the three strains was comparable to the expression level in the wild-type strain (Fig. S4). We then analysed the phenotype of the *fnrKO\_C*, *mapAKO\_C* and *galMKO\_C* strains in the mouse infection model. As shown in Table 3, the three strains were as virulent as the wild-type strain as judged by bacterial load in the blood of the challenged animals.

These results indicate that maltose utilization may represent an important energy source for the *in vivo* growth of *N. meningitidis*.

### Discussion

During infection, bacterial pathogens encounter anatomical compartments with low oxygen levels. In these types of environments, the ability of bacterial pathogens to activate the metabolic shift which allows them to cope with the oxygen-limited conditions is likely to be crucial for their proficient interactions with the host. To investigate on the interplay between anaerobic metabolism and bacterial infection, we have used *N. meningitidis*, a pathogen which appears to be particularly suitable for this kind of studies in that, during infection, it encounters different human compartments where oxygen partial pressure fluctuates quite substantially. In addition, two animal models exist, the infant rat and the adult mouse models (Wilks *et al.*, 1998; Granoff *et al.*, 2001) in which meningococci are administered intraperitoneally and bacterial infectivity is followed by measuring bacteraemia and animal survival. As oxygen-restricted conditions are encountered both in the peritoneum and in the blood, where oxygen is largely sequestered by haemoglobin, these models serve as a good system to examine how specific mutations in genes involved in the adaptation to oxygen restriction affect the virulence potential of *N. meningitidis*.

The first important observation of this study is that FNR inactivation attenuates the virulence of *N. meningitidis* in the two animal models utilized. Apart from the studies on the FNR homologues (Wood *et al.*, 1998; Baltes *et al.*, 2005), this is the first report that demonstrates a direct role of FNR in biological processes that ultimately lead to bacterial survival *in vivo*. FNR is the main regulatory protein responsible for the switch from aerobic to anaerobic metabolism in many bacteria (Guest *et al.*, 1996). In *E. coli*, under anaerobic conditions, the FNR dimer carries two [4Fe-4S] clusters and acquires the capacity to interact with a sequence-specific, promoter-proximal DNA region (FNR box) (Guest *et al.*, 1996). FNR binding results in the upregulation of a set of genes which are involved or linked to bacterial anaerobic functions by virtue of the fact that their expression is enhanced under oxygen limitation. Being FNR a regulatory protein, the observed attenuation of the *fnr* deletion mutant is most likely the result of an altered expression of some anaerobically activated genes. To select these genes,



we defined the *N. meningitidis* FNR regulon, the second FNR regulon so far characterized in bacteria (Salmon *et al.*, 2003; Kang *et al.*, 2005).

The second important piece of information emerging from this study is that in the first 2 h of oxygen starvation, 11 genes, belonging to nine transcriptional units, were upregulated by FNR. The upstream promoter-proximal regions of all nine transcriptional units were able to bind a purified recombinant form of *N. meningitidis* FNR, as judged by gel retardation experiments. Inspection of the nucleotide sequences of these promoter regions allowed us to define a *N. meningitidis* FNR consensus box which appears to be very similar, but not identical to the *E. coli* FNR box (Guest *et al.*, 1996). This is consistent with the fact that the oxygen-resistant *E. coli* FNR D154A mutant (Bates *et al.*, 1995) was capable of binding only five of the nine upstream regions.

The *N. meningitidis* FNR regulon includes the *aniA* (nitrite reductase) and *nosR* (putative nitrous oxide regulator) genes. The *aniA* gene encodes a key gene in anaerobic respiration (Mellies *et al.*, 1997; Householder *et al.*, 1999) and catalyses the reduction of nitrite (NO<sub>2</sub>) to nitric oxide (NO), which is further reduced to nitrous oxide (N<sub>2</sub>O) by the sequential action of a FNR-independent nitric oxide reductase (*norB*) (Householder *et al.*, 2000). Interestingly, from the present study, as well as from our previously published study (Grifantini *et al.*, 2003), it appears that *N. meningitidis* exploits the two major regulatory proteins FNR and Fur to optimize the use of the denitrification pathway. On the one hand, FNR is utilized to upregulate nitrite respiration when oxygen is limiting; on the other hand, as nitrite reductase leads to the accumulation of toxic NO, the organism exploits the capacity of Fur to sense the oxidative stress imposed by NO accumulation and upregulates the expression of the nitric oxide reductase (NorB) (Delany *et al.*, 2004). The most likely explanation why Fur also upregulates *aniA* is that *N. meningitidis* lives in anatomical compartments where both oxygen and iron are limiting. Therefore, the organism utilizes the same regulator (Fur) to simultaneously activate nitrite respiration, iron scavenging and detoxification from NO and iron overload.

The *N. meningitidis* FNR regulon also includes the two transcriptional units NMB0388-*galM* and *mapA-pgmβ*. NMB0388 encodes a putative sugar transporter, while the *galM* gene codes for an aldose 1-epimerase, an enzyme that catalyses the mutarotation of α and β anomers of esoses, such as glucose and galactose. As both anomers are needed in bacteria, aldose 1-epimerase allows the efficient utilization of lactose and galactose whose hydrolysis would otherwise generate only one anomer (Bouffard *et al.*, 1994; Grossiord *et al.*, 2003). MapA is a maltose phosphorylase catalysing the cleavage of maltose to glucose and β-glucose-1-phosphate; that is further converted into glucose 6-phosphate by a β-phosphoglucomutase, encoded by *pgmβ* (Fitting and Dourodoff, 1952; Arai *et al.*, 2003).

The last two genes of the *N. meningitidis* FNR regulon with known function are NMB1805 and NMB1677, which code for cytochrome *c4* and *c5* respectively. In *N. gonorrhoeae*, FNR controls the expression of *ccp*, the cytochrome *c* peroxidase recently reported to be involved in protection of the organism from oxidative stress (Turner *et al.*, 2003). As *ccp* homologues are not present in the *MenB* MC58 genome (Tettelin *et al.*, 2000), it is tempting to speculate that NMB1805 and/or NMB1677 play an analogous protective role against reactive oxygen species.

The remaining three genes of the *N. meningitidis* FNR regulon belong to a hypothetical gene family. NMB0363 encodes a 45-amino-acid-long peptide, while the NMB1806 gene product is homologous to the gonococcal ORF1 and shows significant identity (about 48%) to hypothetical GTP-binding proteins present in several pathogens and sometimes involved in bacterial pathogenicity (van Putten *et al.*, 1998; Zusman *et al.*, 2004). Finally, NMB1870

encodes the surface-associated lipoprotein GNA1870, a recently discovered antigen proposed as an anti-*MenB* vaccine candidate (Massignani *et al.*, 2003).

The third relevant observation of this study is that FNR contributes to the virulence of *N. meningitidis* through the modulation of the expression of at least two transcriptional units, NMB0388-*galM* and *mapA-pgmB*. These genes are involved in maltose catabolism (Qian *et al.*, 1994) and intervene in the first steps of the degradation of simple sugars to form pyruvate. Inactivation of each of these transcriptional units results in the attenuation of *N. meningitidis* in the mouse. In particular, the *galM* and *mapA* knockout mutants were reduced in their ability to survive in the blood by two and three orders of magnitude respectively. The observation that these FNR-dependent genes are important for the survival/proliferation of *N. meningitidis* in the bloodstream of the animals strongly suggests that in this environment where oxygen is limiting the pathogen requires the sugar fermentation pathways for both carbon and energy source.

The possible role of *aniA* in *N. meningitidis* infection deserves our last comment. According to our microarray data, FNR upregulates this gene by more than twofold in oxygen-restricted conditions. Considering that AniA plays a key role in anaerobic respiration, and that the AniA-mediated denitrification pathway and oxygen contribute similarly to neisseral respiration in microaerobic conditions (Rock *et al.*, 2005), one might expect that the observed *in vivo* attenuated phenotype of the *fnr*-deletion mutant is partially ascribable to the insufficient expression of this gene. While we are investigating on this attractive possibility (which seems to be supported by our preliminary data), it has to be pointed out that, based on the genome sequence analysis, *N. meningitidis* seems to have an incomplete denitrification pathway, in that homologous sequences to known N<sub>2</sub>O reductases, which further convert nitrous oxide to dinitrogen, have not been identified (Anjum *et al.*, 2002). This implies that in *N. meningitidis*, less energy is derived from denitrification as compared with bacteria having a complete pathway and would explain why *N. meningitidis* is classified as a strict aerobic organism (Rock *et al.*, 2005). Therefore, the contribution of FNR in *aniA* upregulation might not necessarily influence *N. meningitidis* virulence.

## Experimental procedures

### Bacterial strains, cultures and reagents

Unless differently stated, *MenB* strains were grown on GC broth or GC agar plates (BD Biosciences) supplemented with 4 g l<sup>-1</sup> glucose, 0.1 g l<sup>-1</sup> glutamine, 2.2 mg l<sup>-1</sup> cocarboxylase at 37°C in 5% CO<sub>2</sub>. When necessary, erythromycin (5 µg ml<sup>-1</sup>) was added to the GC medium. Bacteria were inoculated with colonies taken from freshly grown aerobic plates and incubated aerobically to logarithmic phase (OD<sub>600</sub> 0.4) in GC broth and harvested. For aerobic growth, bacteria were resuspended in fresh medium, and 5 ml of aliquots was incubated aerobically at 37°C in 15 ml tubes, shaken at 160 r.p.m. For oxygen-restricted growth, bacteria were resuspended in degassed GC medium and 15 ml of aliquots was incubated in 15 ml sealed tubes. When specified, *MenB* strains were grown aerobically in a chemically defined medium (La Scolea and Young, 1974), containing starch (0.1%) in combination with either glucose (0.75%) or maltose (0.75%) as main carbon sources.

*Escherichia coli* DH5α or BL21 (DE3) was grown aerobically in Luria–Broth (LB) medium (Difco) at 37°C. When appropriate, ampicillin (100 µg ml<sup>-1</sup>), erythromycin (200 µg ml<sup>-1</sup>) and isopropyl-beta-D-galactopyranoside (IPTG, 0.5 mM) were added to the medium. Unless specified, all chemicals used in this study were purchased from Sigma. Restriction enzymes and DNA modification enzymes were from New England Biolabs.

## Mutant preparation and RT-PCR analysis

*fnr*, *mapA*, *galM* null mutants were produced by replacing their entire coding sequences with an erythromycin resistance cassette (*erm*) (Trieu-Cuot *et al.*, 1990). The 500 bp upstream and downstream regions of the gene to be deleted were amplified from MC58 genomic DNA and cloned into plasmid pT7.7 (Amersham Biosciences) on either side of the *erm* gene. The newly derived plasmids were linearized and used for allelic exchange in *MenB*. The resulting mutants, namely MC-*fnr*KO, BZ-*fnr*KO, *map*AKO and *gal*MKO, were verified by PCR of genomic DNA with specific primers.

Complementation of the MC-*fnr*KO, *map*AKO and *gal*MKO deletion mutants was achieved by insertion of the deleted genes (the amplified fragments included the coding regions only) into a non-coding chromosomal location between the two converging ORFs NMB1428 and NMB1429, under the control of the erythromycin resistance gene promoter. This was obtained by cloning the erythromycin resistance gene promoter (Trieu-Cuot *et al.*, 1990) along with and directly upstream of the coding sequence of genes *fnr*, *galM* and *mapA* into the pS1-ComCmr vector (Grifantini *et al.*, 2004). The newly generated plasmids were used to transform MC-*fnr*KO, *map*AKO and *gal*MKO deletion mutants, selecting for chloramphenicol resistance, and the resulting strains *fnr*KO\_C, *map*AKO\_C and *gal*MKO\_C were verified by PCR with specific primers.

For RT-PCR analysis, total RNA (2 µg) was reverse transcribed with random primers and Superscript II Reverse Transcriptase (Invitrogen). The resulting cDNAs were amplified using primer pairs specific for *fnr*, *NMB0388*, *mapA*, *galM*, *pgmB*. RNA-containing PCR reactions, in which the reverse transcriptase step was omitted, were used as controls for DNA contamination. Genomic DNA was amplified as positive control with the same primer sets.

## Microarray procedures, hybridization and analysis

Microarray comparisons were performed on wild-type MC58 and MC-*fnr*KO strains grown in oxygen restriction for 3 h. At time points, total bacterial RNA was extracted from three independent cultures of each sample by using the RNeasy kit (Qiagen, Valencia, CA), pooled and used for cDNA labelling. The hybridization probe was constituted by a mixture of the differently labelled cDNA derived from MC58 and MC-*fnr*KO. Probe hybridization, washing, slide scanning and statistical and clustering analyses were performed as previously described (Grifantini *et al.*, 2003). Genes whose expression ratios changed above twofold and had *P*-values < 0.01 were considered up- or downregulated.

## Computational analysis of FNR binding site

The 400 bp upstream regions of all potential transcriptional units (defined as a cluster of adjacent genes having inter-genic region < 50 bp in length) present in the meningococcal genome were scanned for the presence of conserved sequences using the `FINDPATTERNS` program of the Wisconsin Package (Accelrys, San Diego), under default parameters.

## Purification of FNR proteins from MenB and E. coli and FNR binding studies

The *MenB fnr* gene was PCR-amplified from MC58 chromosomal DNA by using specific primers annealing at the 5' and 3' ends of the gene. *E. coli* D154A *fnr* gene was obtained by PCR from *E. coli* BL21 (DE3) chromosomal DNA. For amplification, two primer pairs were opportunely designed so as to amplify the gene in two partially overlapping fragments, containing the A154 codon within the overlapping region, that were subsequently fused by PCR. The resulting PCR product was verified by DNA sequencing.



Both *E. coli* D154A mutant FNR (Ec FNR) and *MenB* FNR (Nm FNR) proteins were expressed as C-terminal His-tagged fusions by cloning their corresponding amplified genes into pET21b + vector (Novagen), using the *E. coli* BL21 (DE3) strain as the recipient.

Expression of Nm FNR was obtained by growing the recombinant *E. coli* strain in an anaerobic chamber at 25°C. All subsequent steps were carried out as previously described (Lazazzera *et al.*, 1996). Purification of the His-tagged Nm FNR was performed by metal ion affinity chromatography (IMAC) (Amersham Biosciences) in the presence of 1.7 mM sodium dithionite. Expression and purification of Ec FNR was performed aerobically, as described (Ziegelhoffer and Kiley, 1995).

For EMSA on PCR-amplified fragments, 0.025 pmoles of <sup>32</sup>P-ATP-end-labelled promoter fragments were incubated with purified FNR proteins, at a protein dimer concentration ranging from 0 to 800 nM, in the presence of 100 ng of salmon sperm DNA as a specific competitor. For competition experiments, the <sup>32</sup>P-ATP-end-labelled promoter fragments were incubated with Ec FNR (400 nM) and Nm FNR (800 nM) in the presence of variable amounts of each unlabelled DNA fragments. FNR binding was assessed as described (Ziegelhoffer and Kiley, 1995; Lazazzera *et al.*, 1996)

### Animal models

For the mouse model, bacteria were grown overnight on brain–heart infusion (BHI) broth agar plates, then harvested in BHI broth and the number of bacteria was estimated by measuring the OD<sub>600</sub> of the bacterial suspension. Bacteria were harvested by centrifugation, resuspended in BHI medium supplemented with 1% Iron Dextran (w/v) at the desired concentration and 100 µl of suspension was injected in each animal. Six-week-old BALB/c female mice (Charles River laboratories), 10 mice per group, were infected intraperitoneally with 10<sup>7</sup> bacteria of wild-type MC58 or its isogenic mutant strains. Bacterial concentration was confirmed by colony plating. Bacteraemia was assessed by colony plating at 18 h, collecting blood from a tail vein bleed.

The infant rat model was performed as previously described (Granoff *et al.*, 2001). In brief, bacteria were grown to log-phase in Mueller-Hinton (Sigma, St Louis, MO) supplemented with 0.25% glucose, washed and resuspended at the desired concentration in PBS-1%BSA. Four-day-old infant pups from litters of outbred Wistar rats (Charles River, Hollister, CA) were randomly redistributed to the nursing mothers. Groups of six to eight animals were challenged intraperitoneally with bacteria of wild-type BZ232 or its isogenic BZ-*fnr*KO strain. Eighteen hours after the bacterial challenge, blood specimens were obtained by cardiac puncture and blood aliquots were plated onto chocolate agar for viable counting. Standard errors were evaluated by dividing the standard deviation values by the squared root value of number of animals. In the different experiments, standard errors did not vary by more than 1 log the values of the geometrical mean. Statistical difference among groups was evaluated by two-tailed Student's *t*-test. The infant rat studies were approved by the CHORI institutional committee responsible for overseeing experiments in animals.

### Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

### Acknowledgements

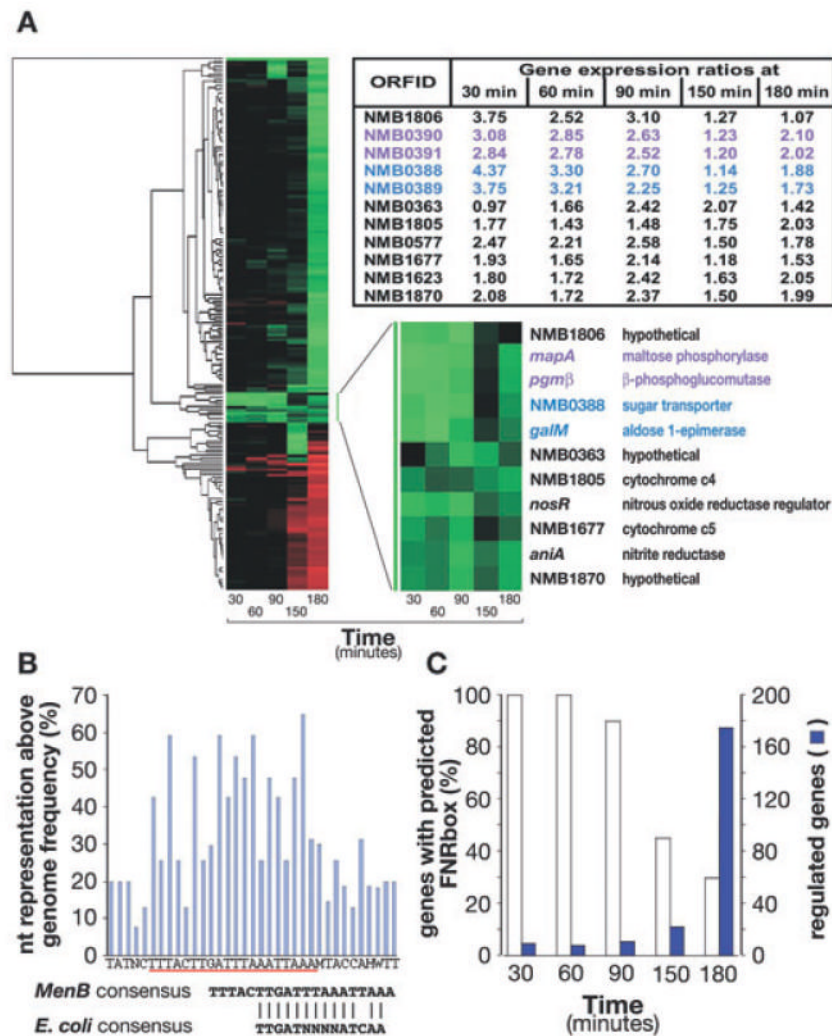
We wish to thank Rino Rappuoli for the useful discussions and the critical reading of the manuscript. We are also grateful to Antonietta Maiorino for her expert secretarial assistance and to Giorgio Corsi for artwork. This work was supported by Public Health Service Grant RO1 AI46464 from the National Institute of Allergy and Infectious Diseases, NIH, and NIH training grant T32-HL007951 (J.A.W.).

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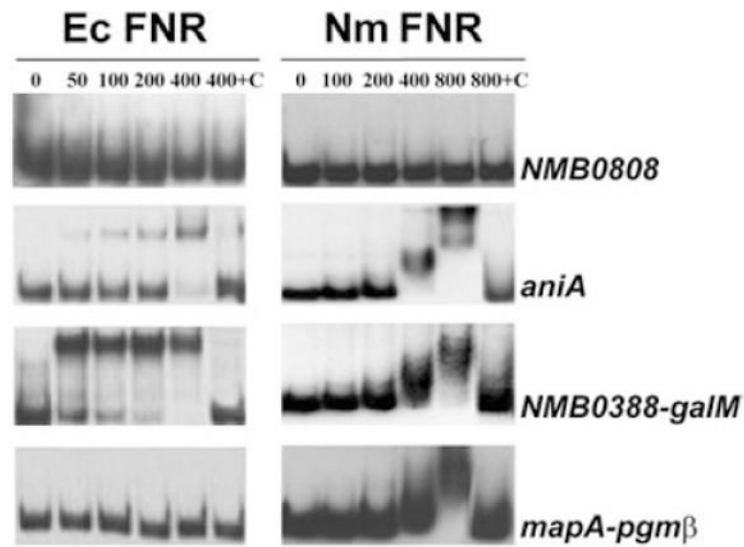
**Fig. 1.** Transcription profile analysis of FNR-dependent genes and computational analysis of *MenB* FNR consensus sequence.

A. Hierarchical clustering of gene expression profiles of wild-type MC58 and MC-*fnr*KO strains grown under oxygen restriction compared by DNA microarray analysis. Red and green bars indicate down- and upregulated genes in the wild-type strain respectively. The zoomed panel represents the upregulated cluster of FNR-dependent genes whose expression ratios are shown in the table. Blue and purple identify genes belonging to the same transcriptional units.

B. Analysis of the FNR consensus sequence derived from the multiple sequence alignment of the upstream regions of the nine clustered upregulated transcriptional units. The graph reports the representation of each nucleotide of the derived consensus sequence above the MC58 genomic nucleotide frequency. The most conserved region of the derived consensus is highlighted in red. Pair-wise alignment of the derived *MenB* FNR consensus with the previously proposed *E. coli* FNR consensus is reported below.

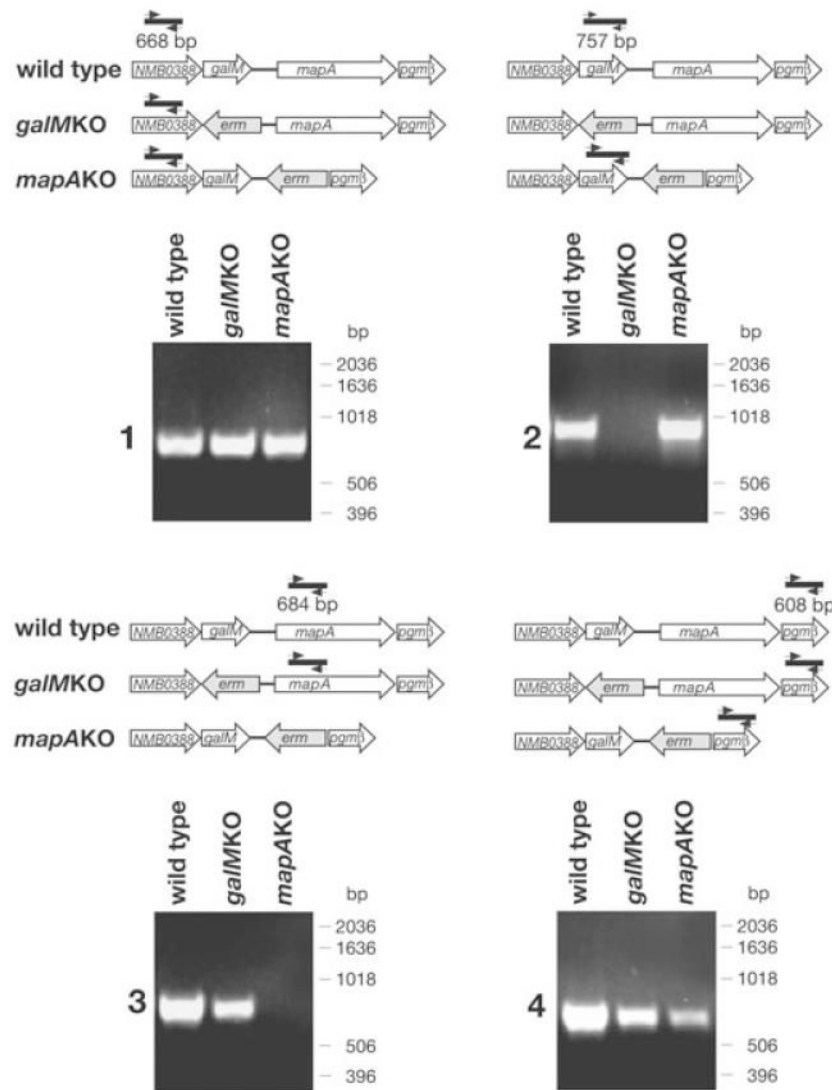
C. Frequency of FNR-regulated genes carrying the FNR consensus sequence. The graph indicates the number of genes (in blue) regulated at different time points and the percentage of regulated genes (in white) with an FNR box in their promoter-proximal region.





**Fig. 2.**

EMSA analysis of FNR-regulated genes. PCR-amplified upstream regions were incubated with different concentrations (nM) of purified Ec FNR and Nm FNR proteins and DNA protein binding was followed by gel retardation analysis in the presence of salmon sperm DNA. To assess FNR binding specificity, reactions containing a 10-fold molar excess of each specific unlabelled DNA region were also included (lanes 400+C and 800+C). NMB0808 was used as negative control as its expression was unaffected by FNR deletion and no FNR consensus sequence was identified in its promoter-proximal region.



**Fig. 3.** Transcription analysis of *galM* and *mapA* genes in wild-type and mutant strains. Total RNA from wild-type and mutant strains was reverse transcribed and the derived cDNAs were amplified using gene-specific primer pairs and analysed by agarose gel electrophoresis. All PCR reactions from non-reverse transcribed RNAs were negative (not shown). The genetic organization of the amplified regions is schematized above each corresponding gel. Arrows indicate the position of the primers used for the amplification reactions and bp numbers indicate the size of each PCR product. Molecular weight standards are given on the right side of each gel.

Table 1

Mouse and infant rat infection assays with wild-type and *fmrKO* strains.

Bacterial strains	Challenge dose	Mouse model		Rat model	
		Number of animals	Bacteraemia <sup>a</sup>	Number of animals	Bacteraemia <sup>a</sup>
MC58 wild type	$4.9 \times 10^7$	50	$1.7 \times 10^5$ *	nd	nd
MC- <i>fmrKO</i>	$5.1 \times 10^7$	50	$9.8 \times 10^3$ *	nd	nd
BZ232 wild type	$4.4 \times 10^3$	nd	nd	6	$3.2 \times 10^6$ **
BZ- <i>fmrKO</i>	$5.0 \times 10^3$	nd	nd	8	$1.4 \times 10^5$ **

<sup>a</sup>Values represent geometric means of the bacterial cfu ml<sup>-1</sup> measured in each animal.

Four-day-old infant rats and 6-week-old BALB/c mice were infected with the indicated amount of bacteria. In the mouse infection model, animals were treated in five independent experiments, 10 mice per group. Bacteraemia was measured 18 h after intraperitoneal challenge.

\*  $P = 0.009$ .

\*\*  $P = 0.06$ .

The significance of differences in the levels of bacteraemia in repeated assays was estimated by two-tailed *t*-test.  
nd, not determined.

**Table 2**

Mouse infection assay with MC58 wild-type, *galMKO* and *mapAKO* strains.

Bacterial strains	Challenge dose	Number of animals	Bacteraemia <sup>a</sup>	<i>P</i> <sup>b</sup>
MC58 wild type	$3.2 \times 10^7$	40	$8.3 \times 10^5$	
<i>mapAKO</i>	$3.6 \times 10^7$	40	$3.9 \times 10^2$	$2.4 \times 10^{-7}$
<i>galMKO</i>	$3.7 \times 10^7$	40	$8.9 \times 10^3$	$2.0 \times 10^{-3}$

<sup>a</sup> Values are geometric means of the bacterial cfu ml<sup>-1</sup> measured in the blood of each animal.

<sup>b</sup> The significance of difference in the level of bacteraemia was evaluated by two-tailed *t*-test.

Six-week-old BALB/c mice, 10 mice per group, were infected with the indicated amount of bacteria in four independent experiments. Bacteraemia was measured 18 h after intraperitoneal challenge.

**Table 3**

Mouse infection assay with MC58 wild-type and the complemented strains *fnr*KO\_C, *gal*MKO\_C and *map*AKO\_C.

Bacterial strains	Challenge dose	Number of animals	Bacteraemia <sup>a</sup>	<i>P</i> <sup>b</sup>
MC58 wild type	$1.7 \times 10^7$	20	$6.3 \times 10^5$	
<i>fnr</i> KO_C	$1.5 \times 10^7$	10	$2.7 \times 10^6$	0.23
<i>map</i> AKO_C	$1.5 \times 10^7$	10	$2.3 \times 10^6$	0.23
<i>gal</i> MKO_C	$1.5 \times 10^7$	10	$2.3 \times 10^6$	0.47

<sup>a</sup>Values are geometric means of the bacterial cfu ml<sup>-1</sup> measured in the blood of each animal.

<sup>b</sup>The significance of difference in the level of bacteraemia was evaluated by two-tailed *t*-test.

Six-week-old BALB/c mice, 10 mice per group, were infected with the indicated amount of bacteria. Bacteraemia was measured 18 h after intraperitoneal challenge.